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Effect of GnRHa ovulation trigger dose on follicular fluid characteristics and granulosa cell gene expression profiles

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Abstract

Purpose A recent dose-finding study showed no significant differences in number of mature oocytes, embryos and top-quality embryos when triptorelin doses of 0.2, 0.3 or 0.4 mg were used to trigger final oocyte maturation in oocyte donors co-treated with a gonadotropin-releasing hormone (GnRH) antagonist. This analysis investigated whether triptorelin dosing for triggering final oocyte maturation in oocyte donors induced differences in follicular fluid (FF) hormone levels and granulosa cell gene expression.

Methods This single-centre, randomised, parallel, investigator-blinded trial was conducted in oocyte donors undergoing a single stimulation cycle at IVFMD, My Duc Hospital, Ho Chi Minh City, Vietnam, from August 2014 to March 2015.

A total of 165 women aged 18–35 years with body mass index <28 kg/m², anti-Müllerian hormone >1.25 ng/mL, and antral follicle count ≥6 were randomised to three different triptorelin doses for trigger. The main outcome was concentration of steroid hormones in FF collected from the first punctured follicle on each side. Moreover, luteinising hormone receptor (*LHR*), 3β-hydroxy-steroid-dehydrogenase (*3βHSD*) and inhibin-Ba (*INHB-A*) gene expression in cumulus and mural granulosa cells were investigated in a subset of women from each group. **Results** Progesterone and oestradiol levels in FF did not differ significantly by trigger doses; findings were similar for *3βHSD*, *LHR* and *INHB-A* gene expression in both cumulus and mural granulosa cells.

Conclusions In women co-treated with a GnRH antagonist, no significant differences in FF steroid levels and granulosa cell gene expression were seen when different triptorelin doses were used to trigger final oocyte maturation.

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Keywords In vitro fertilisation · Gonadotropin-releasing hormone agonist trigger · LHR gene expression · Triptorelin · Follicular fluid

Introduction

Since the introduction of gonadotropin-releasing hormone (GnRH) antagonists for the prevention of a premature luteinising hormone (LH) surge, GnRH agonists (GnRHa) have played an important role in triggering final oocyte maturation [1–4]. Based on a number of advantages, of which the significant reduction of ovarian hyperstimulation syndrome is the most prominent, GnRHa trigger has become the trigger agent of choice for oocyte donors worldwide [5].

Until recently, there were no dose findings studies on GnRHa trigger. However, the results of a recent randomised,

investigator-blinded study showed that there were no significant differences between triptorelin doses of 0.2, 0.3 and 0.4 mg when used for triggering of final oocyte maturation in oocyte donors as regards the number of mature oocytes, top-quality embryos, ovarian volume and luteal phase duration [6]. In addition, there were practically no between-group differences in serum concentrations of LH, follicle-stimulating hormone (FSH), oestradiol and progesterone during the follicular and early luteal phases [6].

The follicular fluid (FF) surrounds the oocyte and its composition is profoundly influenced by the granulosa cell secretions, functioning as a mediator of signalling substances that are transported between cell types within the follicle and the surroundings [7]. In women undergoing assisted reproductive technologies, FF is easily accessible, allowing analysis of hormones and granulosa cell gene expression to determine the impact of ovarian stimulation and the maturation trigger on oocyte maturation.

To further assess the comparability of different triptorelin doses used for trigger [6], the present study (NCT02208986) analysed steroid profiles in FF, and cumulus cell and mural granulosa cells gene expression profiles in GnRH antagonist co-treated women, randomised to three different GnRHa trigger doses.

Materials and methods

This single-centre, randomised controlled trial was conducted from August 2014 to March 2015 at IVFMD, My Duc Hospital, Ho Chi Minh City, Vietnam. All women provided written informed consent to participate in the study, which was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. The Institutional Review Board (IRB) and Ethics Committee approved the study protocol on 21 July 2014 (IRB reference number: NCKH/CGRH_01_2014, and Clinicaltrial.gov registration: NCT02208986). Primary outcome results have been published previously [6].

Subjects

All oocyte donors who met the following criteria were eligible for inclusion in the study: age 18–35 years, body mass index (BMI) <28 kg/m², normal ovarian reserve (anti-Müllerian hormone [AMH] >1.25 ng/mL or antral follicle count [AFC] ≥6 [8] measured within 2 months prior to the start of stimulation), first oocyte donation cycle stimulated with rec FSH and co-treated with a GnRH antagonist, and willingness/ability to comply with protocol requirements for the duration of the study. Exclusion criteria were as follows: polycystic ovary syndrome (PCOS), WHO group 1 anovulation, chronic medical condition (e.g., diabetes, Crohn's disease, thyroid disease, hepatitis B or sexually transmitted diseases), participation in

another clinical trial or concomitant use of either LH or human menopausal gonadotropin (hMG)/urinary FSH preparations during the study cycle.

Stimulation, monitoring and oocyte pick-up

Oocyte donors were randomised to trigger with subcutaneous triptorelin (Ipsen Pharma Biotech, France) at a dose of either 0.2, 0.3 or 0.4 mg. Randomisation was performed using sealed envelopes developed via a computer-generated list with blocks of nine. Physicians, but not women and nurses, were blinded to treatment allocation. Stimulation was performed according to the standard protocol of the unit, using a depot injection of corifollitropin alfa (Elonva®; 100 or 150 µg based on bodyweight) on cycle day 2, followed by co-treatment with a GnRH antagonist (Ganirelix®) and follitropin beta, 150 IU/day from day 8 of stimulation until the day of triggering. When two follicles had reached a size of ≥17 mm, triptorelin was given to trigger final oocyte maturation.

Follicular fluid and hormone level determination

On the day of ovum pick-up, follicular fluid was collected from the first punctured follicle on each side, centrifuged and then cryopreserved at −20°C for subsequent analysis of levels of two important granulosa cell secreted steroids. Progesterone and oestradiol concentrations were measured in individual follicle fluids using commercially available ELISA kits (progesterone: DNOV006, Novatec; 17β-oestradiol: DNO003, Novatec, Immundiagnostica GmbH, Germany). A phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (BSA) was used for dilution of the FF samples prior to measurements according to the manufacturer's instructions. Dilutions of 1:1000 were used for all samples for both progesterone and oestradiol measurements, which resulted in readings inside the standard curve. All samples were run in duplicate and the mean value was used for further calculations. The intra-assay and inter-assay variations were ≤9 and ≤10%, respectively, for oestradiol ELISA, and ≤4 and ≤9.3%, respectively, for progesterone ELISA.

Expression of genes affected by the mid-cycle surge and of importance for the luteal phase

Cumulus and mural granulosa cells from oocyte(s) from the first follicle in the last 10 women recruited to each triptorelin dosage group were isolated immediately after ovum pick-up and snap frozen for analysis of 3β-hydroxy-steroid-dehydrogenase (3βHSD) catalysing conversion of pregnenolone to progesterone, luteinising hormone receptor (*LHR*) stimulating progesterone secretion and inhibin beta A (*INHBA*) gene expression, which becomes heavily upregulated in connection with the mid-cycle surge of gonadotropins. Purification of RNA was

performed using Agilent Absolutely RNA nanoprep kit (Agilent Technologies #400753, Santa Clara, USA). All steps were performed on ice. First-strand cDNA synthesis was carried out using Applied Biosystems High Capacity cDNA reverse transcription kit (Applied Biosystems #4368814, Foster City, USA, lot: 1311191). The synthesis was performed on a thermal cycler (ThermoFisher Scientific Arktik thermal block, Vantaa, Finland) with the following temperature profiles: 25°C for 10 min, 37°C for 120 min, 85°C for 5 s and 4°C until termination. For qPCR, TaqMan Universal PCR master mix (Applied Biosystems, cat. #4304437, Warrington, UK, lot: 1311066) was used as well as the pre-designed TaqMan probes tagged with FAM labelling (Applied Biosystems, Foster City, USA): *LHR* (Hs00174885_m1), *3βHSD* (Hs01084547_gH) and *INHBA* (Hs01081598_m1), plus glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Hs02758991_g1) as housekeeping gene. qPCR reactions were performed in a total volume of 10 µL consisting of a mixture of 0.5 µL 20× TaqMan gene expression assay (TaqMan probe), 0.5 µL 2× TaqMan Universal PCR master mix, 2 µL cDNA and 2.5 µL RNase free water for each reaction. The 96-well qPCR plates (white multiwell plates 96, Roche Diagnostics, cat. #04729692001, Mannheim, Germany) were centrifuged for 1 min at 1000g prior to analysis using the Lightcycler 480 (LightCycler480 II, Roche Diagnostics, Mannheim, Germany). Forty-five cycles of the following PCR program were used: 50°C for 2 min (pre-incubation), 95°C for 10 min, 95°C for 15 s, 60°C for 1 min and finally 40°C for 30 s. All samples were run in duplicates and normalised to *GAPDH* gene expression levels. Calculation of the expression level of the gene of interest was carried out according to the comparative C_T method for relative quantification of gene expression.

Outcome measures

The main outcome measure of this analysis was the level of steroid hormones (oestradiol and progesterone) in FF. Secondary outcome measures were mural and cumulus LHR gene expression.

Statistical analysis

This study was based on a previous randomised trial [6]. Sample size was calculated based on the primary endpoint in the main trial (i.e., determining non-inferiority for the number of MII oocytes in the three different triptorelin dosage groups). The present analysis had a different primary endpoint (steroid hormone levels in follicular fluid). Therefore, we performed a post hoc power calculation [9] to determine whether the sample size of the main trial was sufficient to provide enough power to assess the primary endpoint used in this analysis. The power calculation for one-way analysis of variance tests included the following parameters: number of groups (k) = 3; number of samples in each group = 55; effect size (f) = 0.03;

and α = 0.05. This provided a power of 82% for the current analysis.

Statistical analysis was performed using SPSS 20.0. All tests were two-tailed, and a p value of <0.05 was considered statistically significant. Continuous variables are presented as mean \pm SEM and were tested using analysis of variance with a post hoc test. Categorical data were expressed as numbers and compared, using the Fisher's exact test.

Results

A total of 165 women were enrolled in the study, receiving trigger with either triptorelin 0.2 mg (n = 55), 0.3 mg (n = 55) or 0.4 mg (n = 55). A CONSORT flow diagram is shown in Fig. 1. There were no significant differences between treatment groups in patient characteristics at baseline and overall cycle characteristics (Table 1). In addition, patient characteristics for the 10 women in each group who underwent gene profiling were similar to those of the total study population (Table 1). The effects of the different triptorelin doses on oocyte and embryo outcomes have been reported previously [6]. There were no significant differences between women treated with triptorelin 0.2, 0.3 or 0.4 mg as regards the number of MII oocytes, cleavage rates and the number of top-quality embryos. In addition, the early- and late-luteal-phase steroid profiles were quite similar, and no differences were seen in ovarian volume during the luteal phase [6]. FF analyses were conducted in all 165 enrolled women across the three GnRa trigger dose groups, while gene expression analysis was undertaken in 10 women from each triptorelin dose group.

Follicular fluid hormone levels

Progesterone levels in FF were $48,916 \pm 4011$ nmol/L in the triptorelin 0.2 mg group, $40,240 \pm 3760$ nmol/L in the 0.3 mg group and $48,165 \pm 5093$ nmol/L in the 0.4 mg group, with no significant between-group differences (Fig. 2a). Values for FF oestradiol in the triptorelin 0.2, 0.3 and 0.4 mg groups were also similar, at 1773 ± 260 , 1961.3 ± 335 and 1532 ± 257 nmol/L, respectively (Fig. 2b). Between-group differences in oestradiol and progesterone levels with the upper and lower bounds of the confidence limit confirmed that there were no statistically significant differences between groups in FF steroid hormone levels (Table 2).

Gene expression

The expression of *3β-HSD*, *LHR* and *INHBA* genes in both cumulus cells and mural granulosa cells did not differ significantly after ovulation trigger with either triptorelin 0.2, 0.3 or 0.4 mg (Figs. 3 and 4).

Fig. 1 CONSORT flow diagram. *AMH* anti-Müllerian hormone, *COS* controlled ovarian stimulation, *GnRH* gonadotropin-releasing hormone, *OPU* oocyte pick-up, *PCOS* polycystic ovary syndrome

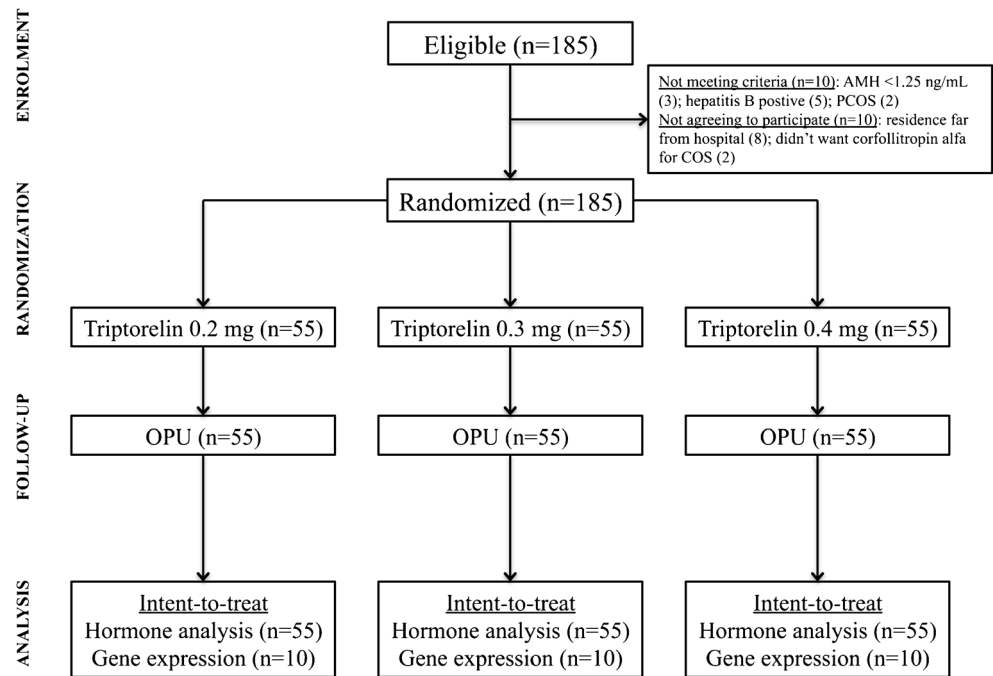


Table 1 Patient and cycle characteristics at baseline

	Triptorelin			<i>p</i> value
	0.2 mg	0.3 mg	0.4 mg	
All patients	(<i>n</i> = 55)	(<i>n</i> = 55)	(<i>n</i> = 55)	
Age, years	27.9 ± 4.6	26.8 ± 3.9	27.1 ± 4.2	0.38 ^a
BMI, kg/m ²	21.1 ± 2.4	20.4 ± 2.8	21.0 ± 2.9	0.89 ^a
AMH, ng/mL	6.8 ± 3.3	7.4 ± 3.3	7.1 ± 3.9	0.62 ^a
AFC, total number	18.0 ± 6.2	19.0 ± 7.3	18.0 ± 6.1	0.97 ^a
Corfolitropin alfa dose, <i>n</i> (%)				0.43 ^b
100 µg	47 (85.5)	51 (92.7)	47 (85.5)	
150 µg	8 (14.5)	4 (7.3)	8 (14.5)	
Duration of stimulation, days	10.4 ± 1.4	10.4 ± 1.5	10.5 ± 1.5	0.69 ^a
Total dose of follitropin beta, IU	540.0 ± 113.6	490.0 ± 155.6	565.5 ± 142.2	0.76 ^a
Patients who underwent gene profiling	(<i>n</i> = 10)	(<i>n</i> = 10)	(<i>n</i> = 10)	
Age, years	27.40 ± 3.75	29.90 ± 4.12	27.00 ± 3.68	0.21 ^a
BMI, kg/m ²	20.87 ± 1.70	20.59 ± 1.34	21.68 ± 2.95	0.50 ^a
AMH, ng/mL	6.49 ± 2.94	6.00 ± 3.28	8.59 ± 4.07	0.23 ^a
AFC, total number	13.60 ± 4.12	14.60 ± 7.62	19.70 ± 5.29	0.06 ^a
Corfolitropin alfa dose, <i>n</i> (%)				0.29 ^b
100 µg	9 (90.0)	10 (100.0)	7 (70.0)	
150 µg	1 (10.0)	0 (0.0)	3 (30.0)	
Duration of stimulation, days	9.90 ± 1.20	10.50 ± 1.72	10.10 ± 1.97	0.72 ^a
Total dose of follitropin beta, IU	685.00 ± 333.37	850.00 ± 471.99	695.00 ± 451.20	0.63 ^a

Data are presented as mean ± standard deviation or number of patients (%)

AFC antral follicle count, AMH anti-Müllerian hormone, BMI body mass index

^a Analysis of variance

^b Fisher's exact test

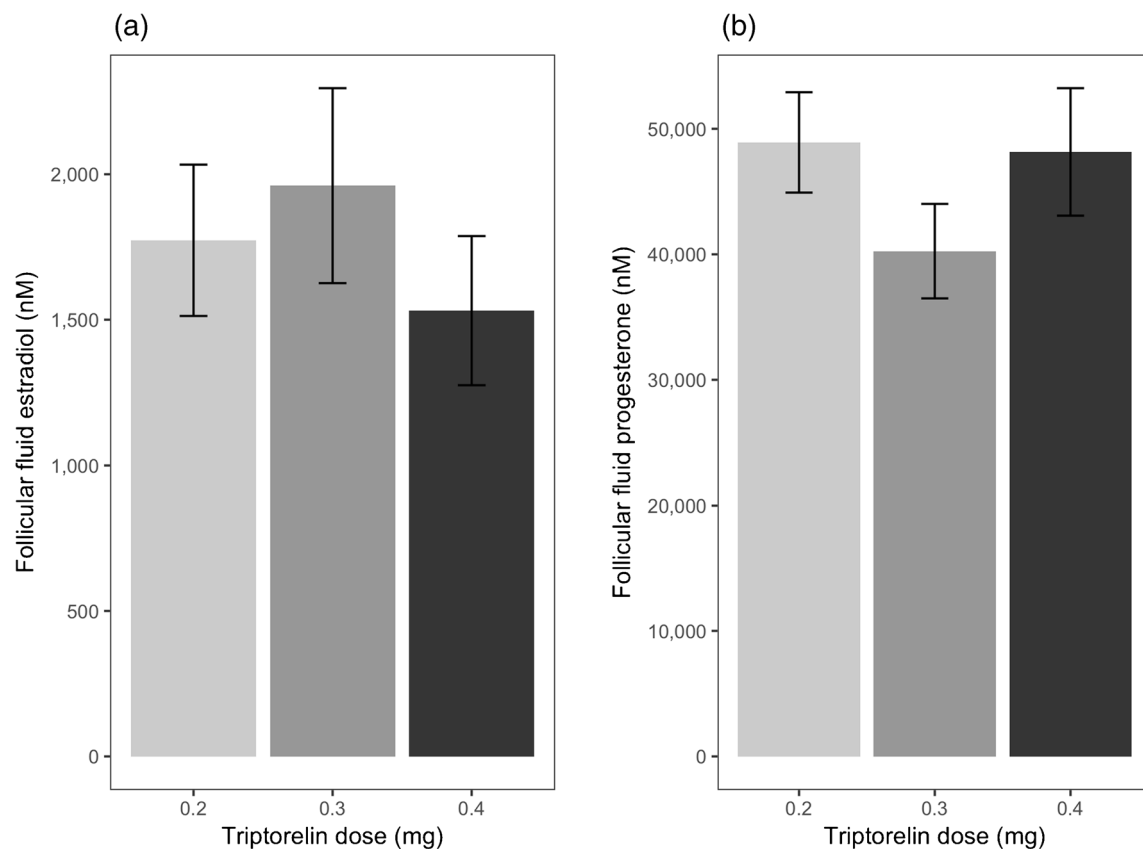


Fig. 2 Hormone levels (**a** progesterone and **b** oestradiol) in follicular fluid after trigger using triptorelin 0.2, 0.3 and 0.4 mg

Discussion

This study showed no differences in FF steroid hormone levels and no differences in either mural or cumulus cell gene expression profiles in human follicles triggered with one of three different doses of triptorelin (0.2, 0.3 or 0.4 mg). This confirms and extends previous clinical results showing similar outcomes with respect to oocyte number, maturity and quality, regardless of the GnRHa trigger dose used [5, 6].

The fact that the GnRHa-induced gonadotropin surge successfully induces full oocyte maturation although the surge is

of shorter duration and with a significant difference in profile compared with the natural mid-cycle surge of gonadotropins [10, 11] demonstrates that the threshold level of LH-like activity required to induce oocyte maturation is lower than seen during natural cycle.

These results are in accordance with data from the rat model in which it was shown that ovulation per se requires an LH-like activity of around 85% of the natural mid-cycle surge of gonadotropins, whereas oocyte maturation occurs when follicles are exposed to around one third of the natural mid-cycle surge of gonadotropins [12]. As a consequence, the use of an

Table 2 Between-group differences in oestradiol and progesterone levels

	Mean difference between doses	95% confidence limit		Adjusted <i>p</i> value
		Lower	Upper	
Oestradiol level				
Triptorelin 0.3–0.2 mg	188.34	−765.98	1142.66	0.887
Triptorelin 0.4–0.2 mg	−241.11	−1204.56	722.34	0.825
Triptorelin 0.4–0.3 mg	−429.45	−1392.90	534.01	0.544
Progesterone level				
Triptorelin 0.3–0.2 mg	−8675.74	−22996.97	5645.49	0.326
Triptorelin 0.4–0.2 mg	−751.49	−15,209.77	13,706.79	0.992
Triptorelin 0.4–0.3 mg	7924.25	−6534.03	22,382.53	0.399

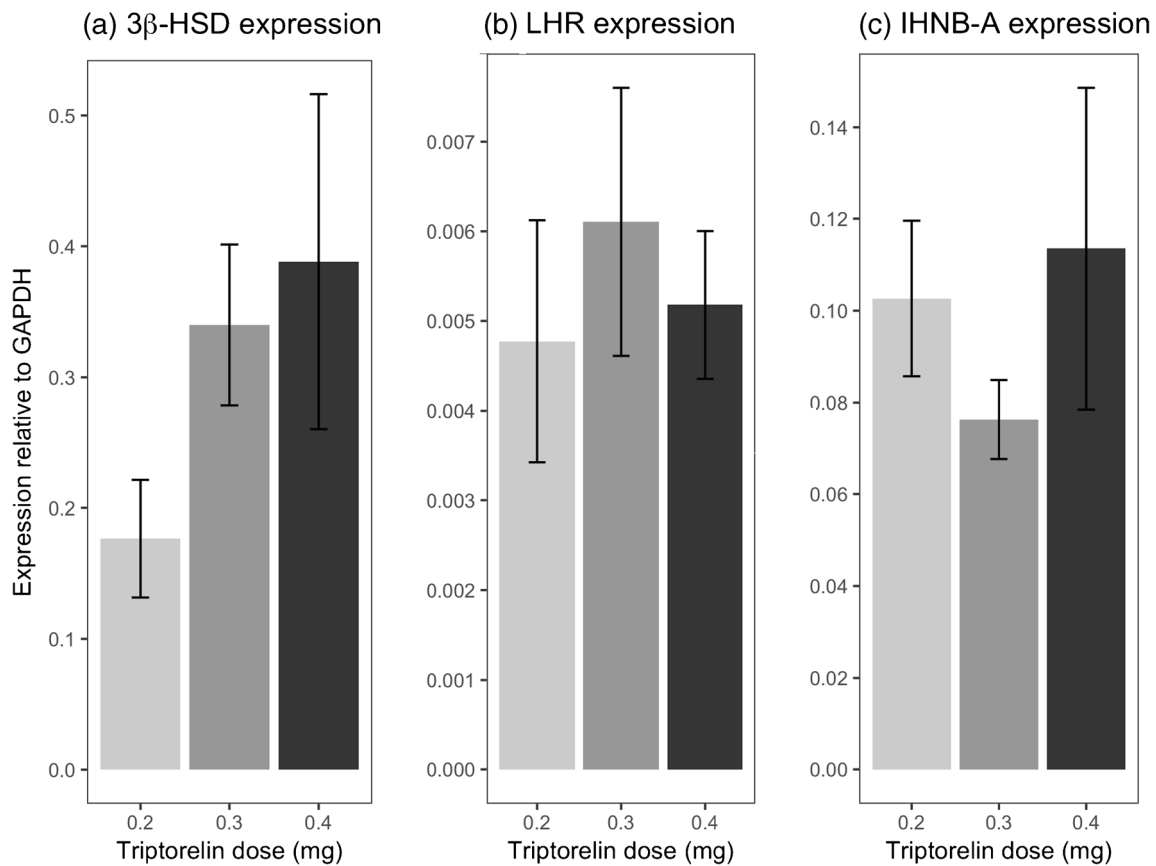


Fig. 3 Gene expression in cumulus cells, normalised to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), after trigger using triptorelin 0.2,

0.3 and 0.4 mg. **a** 36-Hydroxysteroid dehydrogenase (*HSD*). **b** Luteinising hormone receptor (*LHR*). **c** Inhibin beta A (*IHNB-A*)

hCG trigger, e.g., 10,000 IU, which is considered the gold standard, characterised by an extended half-life and supra-physiological levels of LH-like activity, will provide a signal for final maturation of oocytes which by far exceeds the amount necessary [13].

Collectively, the present results suggest that final maturation of oocytes, using an endogenously released mid-cycle surge, is relatively insensitive to the dose of GnRHa used and that even a relatively small bolus of GnRHa is capable of inducing oocyte maturation in women with the same characteristics as our study population. Moreover, the data corroborate previous research, supporting the idea that different types of GnRHa and different pharmacological doses of GnRHa may be used for final maturation of oocytes without negatively impacting the oocyte maturation rate and the oocyte quality [5].

In an early study showing that the FF fluid microenvironment prior to ovulation is dependent on the method by which ovulation is triggered, concentrations of inhibin and progesterone in FF were significantly higher after triggering with hCG versus GnRHa [14]. Subsequent studies confirmed and extended these findings, with higher FF progesterone and amphiregulin levels seen after hCG trigger compared with

GnRHa trigger [15, 16]. Moreover, significant differences in early luteal endometrial gene-expression profiles were seen when comparing hCG trigger to GnRHa trigger [17]. Furthermore, differences between GnRHa and hCG trigger have been documented for gene expression profiles in granulosa and cumulus cells, with LH suggested to be the upstream regulator responsible for these observations [18]. Differences are likely to reflect that a standard hCG trigger induces supra-physiological levels of LH-like activity which boosts ovarian steroidogenesis in a more pronounced way than a GnRHa trigger. In contrast, the GnRHa trigger has a reduced stimulatory effect on steroidogenesis as compared to that of the natural mid-cycle surge of gonadotropins; however, full oocyte maturation is still achieved. Importantly, the risk of ovarian hyperstimulation syndrome (OHSS) is significantly lower after GnRHa compared with hCG trigger [5, 6, 19–21].

Based on the equivalence of the triptorelin doses studied with respect to oocyte number, maturity and quality [6], the lack of a significant difference between dose groups with respect to FF steroid hormone levels is not unexpected. However, variations in gene expression warrant further investigation in larger patient groups. Although our data from 10 patients showed no significant

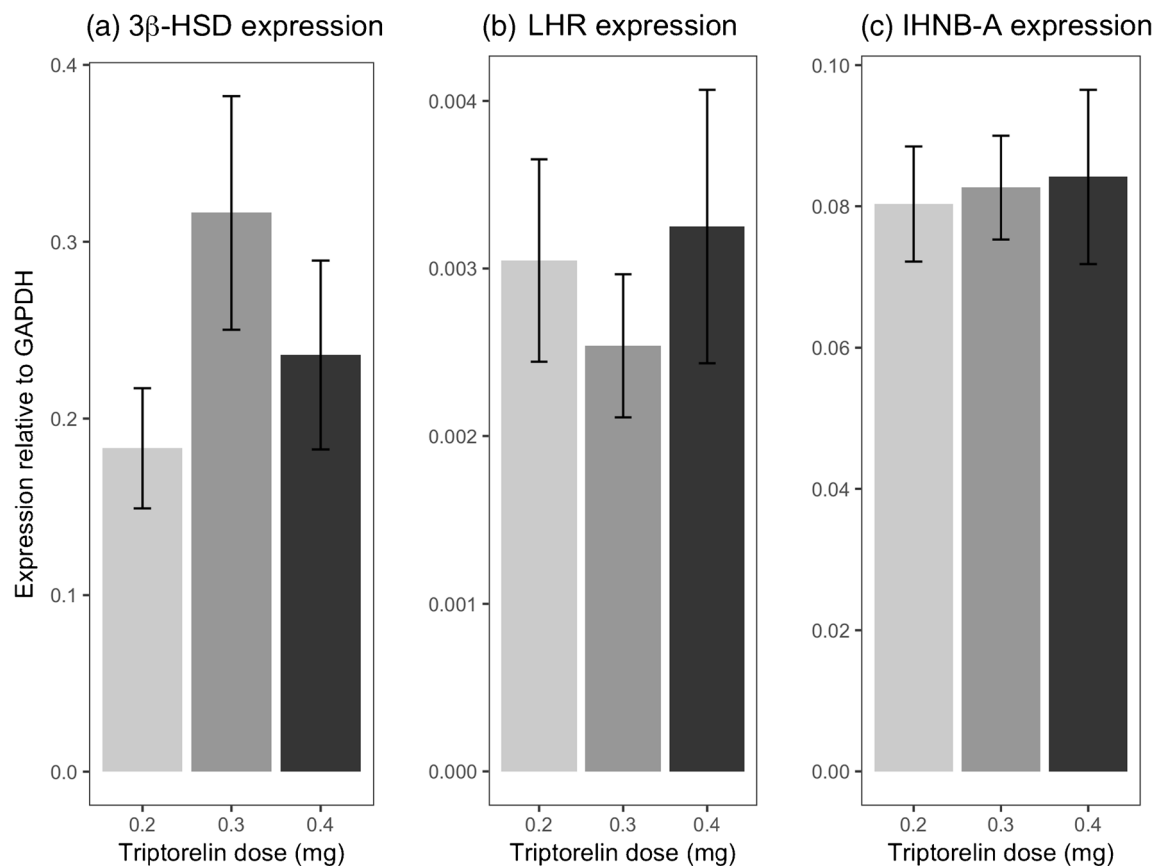


Fig. 4 Gene expression in mural granulosa cells, normalised to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), after trigger using triptorelin 0.2, 0.3 and 0.4 mg. **a** 3β-Hydroxysteroid dehydrogenase (*HSD*). **b** Luteinising hormone receptor (*LHR*). **c** Inhibin beta A (*IHNB-A*)

between-group differences, values were not always consistent across triptorelin doses, suggesting that gene expression in cumulus and mural granulosa cells may be more sensitive to the GnRHa trigger dose.

Limitations of the present study are that the analysis was performed in an Asian population only, and that the GnRHa trigger doses studied theoretically may not be applicable to other clinical settings and countries; moreover, the inclusion of oocyte donors only limits the ability to generalise the results to a wider population of women undergoing infertility treatment. Finally, the gene expression analysis was performed in a subset of women from each dosing group (10 for each triptorelin dose), potentially reducing the power of this analysis. Although we did not find statistically significant between-group differences, some dose-dependent trends in gene expression can be seen in the figures, particularly for HSD gene expression in mural granulosa cells (Fig. 4a). Therefore, it is possible that these could reach statistical significance if analysed in a larger group of patients. Nevertheless, the current data corroborate previous clinical findings [6], which combined provide a complete picture of the effects of the different GnRHa trigger doses studied.

In conclusion, no significant differences in follicular fluid levels of steroid hormones and granulosa cell gene expression were seen in oocyte donors co-treated with a GnRH antagonist when triptorelin doses ranging from 0.2 to 0.4 mg were used to trigger final oocyte maturation. These findings, and results in the same patients showing no influence of triptorelin dose on oocyte number, maturity and quality, favour the use of the lower triptorelin trigger dose.

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Compliance with ethical standards

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Competing interests The authors state that they have no competing interests.

Ethics approval and informed consent All patients provided written informed consent. The study protocol was approved by the Institutional Review Board (IRB) and Ethics Committee on 21 July 2014 (IRB reference number: NCKH/CGRH_01_2014).

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